Arabidopsis thaliana **plastoglobule-associated fibrillin 1a interacts with fibrillin 1b** ***in vivo***

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Abstract

Plant fibrillins are a well-conserved protein family found in the plastids of all photosynthetic organisms, where they perform a wide range of functions. A number of these proteins have been suggested to be involved in the maintenance of thylakoids and the formation of plastoglobules, preventing their coalescence and favouring their clustering via an as-yet unidentified cross-linking mechanism. In this work we show that two members of this group, namely fibrillin 1a and 1b, interact with each other via a head-to-tail mechanism, thus raising the possibility that they form homo- or hetero-oligomers and providing a mechanism to understand the function of these proteins.

Keywords

Fibrillin, plastoglobules, Arabidopsis, protein-protein interaction

Abbreviations

FBN, fibrillin; Y2H, yeast Two-Hybrid; BiFC, bimolecular fluorescence complementation; CTP, chloroplast transient peptide

Highlights

- An interaction between plastidial proteins fibrillin 1a and 1b is shown
- Fibrillin 1a-Fibrillin 1a and Fibrillin 1b-Fibrillin 1b interactions are also shown
- Interaction takes place via a head-to-tail mechanism
- These interactions may explain the functions of these proteins in the plastids

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Introduction

The term fibrillins (FBNs) in plants designates a large protein family that is present in all types of plastids, such as chloroplasts, chromoplasts, and leucoplasts (Eugeni Piller et al., 2012). These proteins were named fibrillins as they were first identified in fibrils, the thread- or tube-like structures found in bell pepper (*Capsicum annuum*) fruit chromoplasts (Deruere et al., 1994), which are the main sites of chromoplasts for pigment accumulation (Winkenbach et al., 1976). Fibrillins are found in all photosynthetic organisms, ranging from cyanobacteria to plants (Laizet et al., 2004). Most of them are located in plastoglobules (Ytterberg et al., 2006), although the presence of FBNs in the stroma and stromal lamellae thylakoids has also been shown (Eymery and Rey, 1999). FBNs can be divided into 12 phylogenetic groups and appear to be involved in a wide range of functions, such as abiotic stress tolerance, growth and development, ~~and~~ hormone signalling, or lipid transport between thylakoid membranes and plastoglobules (Simkin et al., 2007; Singh and McNellis, 2011). Members of group 1, which includes *Arabidopsis* FBN1a and FBN1b, have been reported to be involved in plastoglobule formation and thylakoid maintenance (Rey et al., 2000; Simkin et al., 2007). Indeed, a recent proteomics study has indicated that FBNs are the most abundant proteins in the plastoglobules of *Arabidopsis* leaf rosettes, and six FBNs, including the four major ones (FBN1a, 1b, 2 and 4), account for 53% of the plastoglobule protein mass in *Arabidopsis* (Lundquist et al., 2012). The addition of a bell pepper FBN orthologous to *Arabidopsis* FBN1a to carotenoids and polar lipids in the same stoichiometric ratio found *in vivo* reconstituted the fibrils observed in bell pepper fruit chromoplasts (Deruere et al., 1994), and over-expression of this FBN led to

an increase in the number of plastoglobules organized into clusters in tobacco chloroplasts (Rey et al., 2000). In light of these results, it has been hypothesized that FBN may prevent plastoglobule coalescence and favour their clustering by acting as an interface between the aqueous environment and lipids as well as by mediating cross-linking via an unknown mechanism (Rey et al., 2000). In this work we demonstrate an interaction between *Arabidopsis* FBN1a and FBN1b proteins, as well as FBN1a-FBN1a and FBN1b-FBN1b interactions. Yeast Two-Hybrid analyses indicate that these interactions involve the N-terminal part of one protein and the C-terminal part of the other in a head-to-tail mechanism, thereby suggesting the possibility that these proteins form filaments *in vivo*. These interactions provide a mechanism to explain the function of these FBNs in fibril formation or the maintenance of plastoglobule clusters.

Materials and Methods

Plasmid construction. Full-length ORFs from FBN1a and FBN1b were cloned (without the stop codon) into the pDONR221 entry vector (Invitrogen) by a BP reaction (Invitrogen). After sequence verification, the inserts were transferred into the binary vectors pXNGW (-*n*YFP) and pXCGW (-*c*CFP) (Yuan et al., 2013), respectively, for the BiFC assay using LR Clonase II (Invitrogen). This resulted in translational fusions between the ORFs and the YFP/CFP moieties driven by the CaMV 35S promoter.

For Y2H screening, a cDNA fragment coding for amino acids 71 to 144 of FBN1b was amplified from *Arabidopsis* cDNA using the primers FBN1B_Y2H_F

and FBN1b_Y2H_R (see list of primers in Table S1). These primers introduced *Nde*I and *EcoR*I restriction sites at the 5'- and 3'-ends of the fragment, respectively. The fragment amplified was cloned into the yeast vector pGBKT7 (Clontech) by restriction with *Nde*I and *EcoR*I and subsequent ligation.

For the BiFC assays of the N- and C-terminal part of FBN1b, the cDNA fragment coding for the FBN1b chloroplast transit peptide (CTP) was fused to the fragment coding for amino acids 71 to 144 by two sequential steps of PCR amplifications using cDNA as probe and primers FBN1b_CTP-bait_F and FBN1b_CTP-bait_R in the first step, and the products of the first PCR amplification and primers FBN1b_F and FBN1b_CTP-bait_GW_R in the second step (see primers list in Table S1). The C-terminal part of FBN1b (amino acid residues 220 to the end) was fused to the CTP region using the same strategy with the primers FBN1b_CTP-Ct_F and FBN1b_CTP-Ct_R in the first PCR amplification and FBN1b_F and FBN1b_R in the second step. Once the N- and the C-terminal regions of FBN1b had been fused to the CTP, both constructs were cloned into the binary vectors pXNGW (-*n*YFP) and pXCGW (-*c*CFP), respectively, using LR Clonase II (Invitrogen).

The full-length ORF of VTE1 (At4g32770) gene was amplified from leaves mRNA by RT-PCR using the primers described in Table S1, and cloned into the pDONR221 entry vector (Invitrogen) by a BP reaction (Invitrogen). After sequence verification, the insert was transferred into the binary vectors pXNGW (-*n*YFP) and pEarlyGate103 (Earley, et al., 2006), for BiFC assays and fusion to GFP marker respectively.

Transient expression in *Nicotiana benthamiana*. The corresponding vectors were electroporated into *Agrobacterium tumefaciens* strain C58 (Wood et al., 2001). The saturated overnight bacterial cultures carrying the GFP or the YFP/CFP construct moieties were each adjusted to a final $O.D._{600nm}$ of 0.2 and then co-infiltrated with equal amounts of an *Agrobacterium* suspension carrying a p19 suppressor of post-transcriptional gene silencing, following the method of Silhavy et al. (Silhavy et al., 2002). The *Agrobacterium* suspensions were then infiltrated into the leaves of three- to four-week-old *N. benthamiana* plants as described previously (Marillonnet et al., 2005). The infiltrated plants were kept in a controlled growth chamber under the above conditions for two days until analysis by confocal microscopy.

Yeast Two-Hybrid screening. Screening of a commercial, normalized, *Arabidopsis* cDNA library in the yeast expression vector pGADT7-RecAB (Clontech) was performed using the Matchmaker Gold Yeast Two-Hybrid system (Clontech), following the manufacturer's instructions. The bait used in the screening was a fragment corresponding to the N-terminal part of FBN1b (amino acid residues 71 to 144). The cDNA encoding for the bait was cloned into the yeast cloning vector pGBKT7 as described above.

Confocal microscopy. A DM6000 confocal laser scanning microscope (Leica Microsystems) equipped with a 63x water-immersion objective was used to examine protein-protein interactions in BiFC assays involving *N. benthamiana* mesophyll cells. YFP/CFP expression and chlorophyll autofluorescence imaging

was performed by excitation with a 488 nm argon laser; fluorescence was detected at 500-525 and 630-690 nm, respectively.

Results and Discussion

Searching for proteins that interact with FBN1b using the yeast two-hybrid (Y2H) system. Previous studies from our group have identified the interaction of FBN1b with a protein involved in starch metabolism (unpublished results). In order to continue with this analysis, we searched for other proteins that could interact with FBN1b by screening an *Arabidopsis* cDNA library using the Y2H system and a fragment of the N-terminal part of FN1b (residues 71 to 144) as bait. We selected this fragment because it contains a hydrophilic domain (corresponding to Block 1) defined by Laizet et al. (2004) and may therefore be involved in functions distinct from the interaction with the lipids of plastoglobules, such as protein-protein interactions.

The cDNA fragment coding for the FBN1b fragment was fused to the DNA binding domain of the yeast GAL4 transcription factor by cloning into the expression vector pGBKT7 and subsequent transformation into the yeast strain Y2HGOLD (Materials and Methods). Expression of this construct was confirmed by immunoblotting yeast crude extracts employing polyclonal antibodies against the GAL4 DNA binding domain (data not shown). The fragment was used to screen a normalized, commercial *Arabidopsis* cDNA library in the pGADT7-Rec yeast expression vector (Clontech). Although 35 positive clones that grew on selective medium were obtained, 22 of these encoded for proteins with an

extra-plastidial localization and were therefore discarded. The remaining proteins found are listed in Table 1. We decided to focus on the study of the positive clones encoding for fragments of FBN1a and FBN1b proteins as these results suggest that FBN1b can interact with another FBN1b polypeptide to form homodimers or with FBN1a to form heterodimers.

FBN1b interacts with FN1a and FBN1b *in vivo*

We decided to confirm the interactions observed with the Y2H system using bimolecular fluorescence complementation (BiFC) during transient expression of selected proteins in *Nicotiana benthamiana* leaves. Full-length cDNA sequences from At4g04020 (FBN1a) and At4g22240 (FBN1b) were cloned into the binary vector pXCGW (-*c*CFP) and subjected to *Agrobacterium*-mediated co-transformation in *N. benthamiana* together with the FBN1b cDNA cloned into pXNGW (-*n*YFP). At the same time we determined the localization pattern of over-expressed FBN1b fused to GFP and transiently expressed in *N. benthamiana* leaves. Figure 1 shows that FBN1b over-expressed in *N. benthamiana* leaves is mainly located in specific regions of the chloroplasts. The same result was obtained when *Arabidopsis* FBN1a fused to streptavidin and to HIVp24 was over-expressed in transplastomic tobacco chloroplasts (Shanmugabalaji et al., 2013). Over-expression of an FBN1a ortholog of bell pepper in tobacco led to an increased number of plastoglobules organized in clusters (Rey et al., 2000), which could explain the appearance of over-expressed FBN1b (mainly located in plastoglobules) in specific regions of *N. benthamiana* chloroplasts. Confocal microscopy analysis confirmed that FBN1b interacts with both FBN1b and FBN1a in such chloroplasts (Figure 2), exhibiting

a localization pattern that coincides with that shown by the FBN1b-GFP fusion in both cases (Figure 1). Both FBN1a and FBN1b are over-expressed in *Nicotiana* leaves during the BiFC assays, which raise the question whether the observed interaction between both proteins could be an artefact due to their over-expressions together with their localization limited to such small bodies as the plastoglobules. To address this question we tested the possible interaction between FBN1b and another plastoglobule-associated protein under our BiFC assay conditions. We selected the tocopherol cyclase enzyme (VTE1), encoded by the At4g32770 gene, which is associated to plastoglobules (Vidi et al, 2006). cDNA coding sequence for VTE1 was cloned into the binary vector pXNGW (-*n*YFP) and co-transformed in *N. benthamiana* leaves together with FBN1b cloned into pXCGW (-*c*CFP). Figure S1 shows that there is not interaction between VTE1 and FBN1b proteins (Figure S1, Panels A and D), whereas fluorescence was detected when, in the same experiment, *Nicotiana* leaves were co-transformed with FBN1a and FBN1b proteins or VTE1 fused to GFP (Figure S1, Panels G and J respectively). These results indicate that the interaction between FBN1a and FBN1b proteins observed using the BiFC technique is not a mere consequence of the over-expression of two proteins whose localization is restricted to a small area, such as plastoglobules surface, and support the idea that FBN1a and FBN1b interact *in vivo* in the plant.

Analysis of the domains involved in the interactions

The different FBN1a and FBN1b clones obtained in the Y2H screening were used to identify the domains involved in the interactions between these proteins. The lengths of the clones obtained are illustrated in Figure 3. According to this

alignment, the FBN1b region extending from amino acid 220 to the C-terminal end at position 322 is sufficient to interact with the fragment used as bait. To confirm this we used a BiFC assay in *Nicotiana benthamiana* leaves. The FBN1b fragment used as bait in the Y2H screening was fused to the chloroplast transit peptide of FBN1b and cloned into the binary vector pXNGW (-*n*YFP). The C-terminal part of FBN1b (residues 220 to 322) was also fused to the FBN1b CTP and cloned into pXCGW (-*c*CFP). Both constructs were agro-infiltrated in *N. benthamiana* leaves and fluorescence monitored by confocal microscopy. Figure 4 shows that the N- and C-terminal fragments of FBN1b used in the assay interact *in vivo*.

In the case of FBN1a clones, the minimum fragment that interacts with the FBN1b bait extends from amino acid 125 to the C-terminal end of the protein (Figure 3). The minimum domain delimited for FBN1b (residue 220 to the stop codon) differs from the equivalent region of FBN1a by five non-conserved amino acid changes, thus suggesting that the FBN1a domain involved in the interaction with the bait might also be delimited to the region expanding from amino acid 220 to the C-terminal end of the polypeptide. These regions contain one of the hydrophobic domains (H3) described for FBN7 (see Figure S2), which could be involved in anchoring fibrillins to plastoglobules (Vidi et al., 2007). Once this domain, which would not be involved in protein-protein interactions, has been eliminated, the region responsible for the interaction between FBN1a and FBN1b might be delimited to the fragment expanding from amino acid 220 to 275 (Figure S2).

These results indicate that FBN1a and FBN1b can interact through a head-to-tail mechanism. Moreover, experimental evidence suggests that fibrillins of

Group 1 (which includes FBN1a and FBN1b) are involved in plastoglobule formation (Rey et al., 2000) and thylakoid maintenance (Simkin et al., 2007), and it has been suggested that these proteins, being an interface between the aqueous phase and lipids as well as mediating cross-linking by an unknown mechanism, may prevent plastoglobule coalescence and favour their clustering by mediating interactions between plastoglobules. Our results provide a mechanism for these FBNs to carry out their function. Thus, the head-to-tail interaction would allow the formation of a network that could contribute to maintaining the plastoglobule structure and their association to form clusters. Our results also indicate that, in addition to the FBN1a-FBN1a and FBN1b-FBN1b interaction, FBN1a can interact with FBN1b in *N. benthamiana* chloroplasts, thereby raising the possibility of heterodimer or heteropolymer formation *in vivo*. The high homology between FBN1a and -1b sequences and their exon/intron organization (both genes have two introns at the same position) suggest that they were originated by recent gene duplication. However, expression of these genes does not respond to the same external stimuli; FBN1a expression is induced in cold-stressed *Arabidopsis* plants, whereas FBN1b expression remains unaffected (Laizet et al., 2004). Thus, the formation of dimers or polymers containing both polypeptides could vary depending on the levels of gene expression in response to environmental conditions. The region of FBN1a and -1b that interacts with the bait used in the Y2H screening is not well conserved among the remaining fibrillins (Figure S2), thus suggesting that other FBNs do not interact with FBN1a or FBN1b. It will therefore be interesting to determine whether other FBNs display a similar protein-protein

interaction mechanism and the effect of these possible interactions on their respective functions.

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Figure Legends

Figure 1. **Localization of FBN1b fused to GFP in *Nicotiana benthamiana* chloroplasts**. cDNA coding for the full-length FBN1b protein was fused to GFP protein and transiently expressed in *N. benthamiana* leaves. A: GFP fluorescence. B: chlorophyll autofluorescence. C: combination of images A and B.

Figure 2. Interaction between FBN1b and FBN1a. cDNAs coding for the full-length FBN1b (Panels A, B and C) and FBN1a proteins (Panels D, E, and F) were fused to the N-terminal half of YFP and co-transformed in *N. benthamiana* leaves together with the cDNA coding for FBN1b fused to the C-terminal moiety of CFP. A and D correspond to YFP/CFP fluorescence and B and E to chlorophyll autofluorescence. C and F are combinations of images A and B, and D and E, respectively.

Figure 3. FBN1a and FBN1b clones found in Y2H screening. The amino acid sequences of the different clones of FBN1a and FBN1b identified in the Y2H screening of an *Arabidopsis* cDNA library are aligned with the FBN1a and FBN1b polypeptides. The initial amino acid of each clone is indicated by a number. Clones of the FBN1b gene are indicated by light grey boxes; dark grey boxes indicate FBN1a clones. Black boxes indicate the sequences encoding for the chloroplast transit peptide of both genes. The FBN1b fragment used as bait in the Y2H screening is indicated. The minimal fragment of FBN1a and FBN1b necessary to interact with the bait is delimited by two vertical parallel lines.

Figure 4. Interaction between the N- and C-terminal parts of FBN1b. The cDNA fragment of FBN1b gene used in the Y2H screening (amino acids 71 to 144) was fused to the FBN1b CTP region and to the N-terminal half of YFP and co-transformed in *N. benthamiana* leaves together with the cDNA coding for the C-terminal part of FBN1b determined previously (amino acids 220 to the end) (see Figure 3) fused to the FBN1b CTP region and to the C-terminal moiety of CFP. A: YFP/CFP fluorescence. B: chlorophyll autofluorescence. C: combination of images A and B. A scheme of both constructs is indicated. N-

FBN1b: N terminal part of FBN1b. C-FBN1b: C-terminal part of FBN1b. Numbers indicate amino acid positions in the FBN1b polypeptide.

Table

Table 1. **Plastidial proteins found in Y2H screening of an *Arabidopsis* library using the N-terminal part of FBN1b as bait**

Number of independent clones	AGI Code	Protein
1	Atcg00810	Ribosomal protein L22
1	At4g04640	ATPase, F1 complex, gamma subunit protein
1	At3g04790	Ribose 5-P isomerase
2	At4g04020	Fibrillin1a
4	At4g22240	Fibrillin1b

Figure

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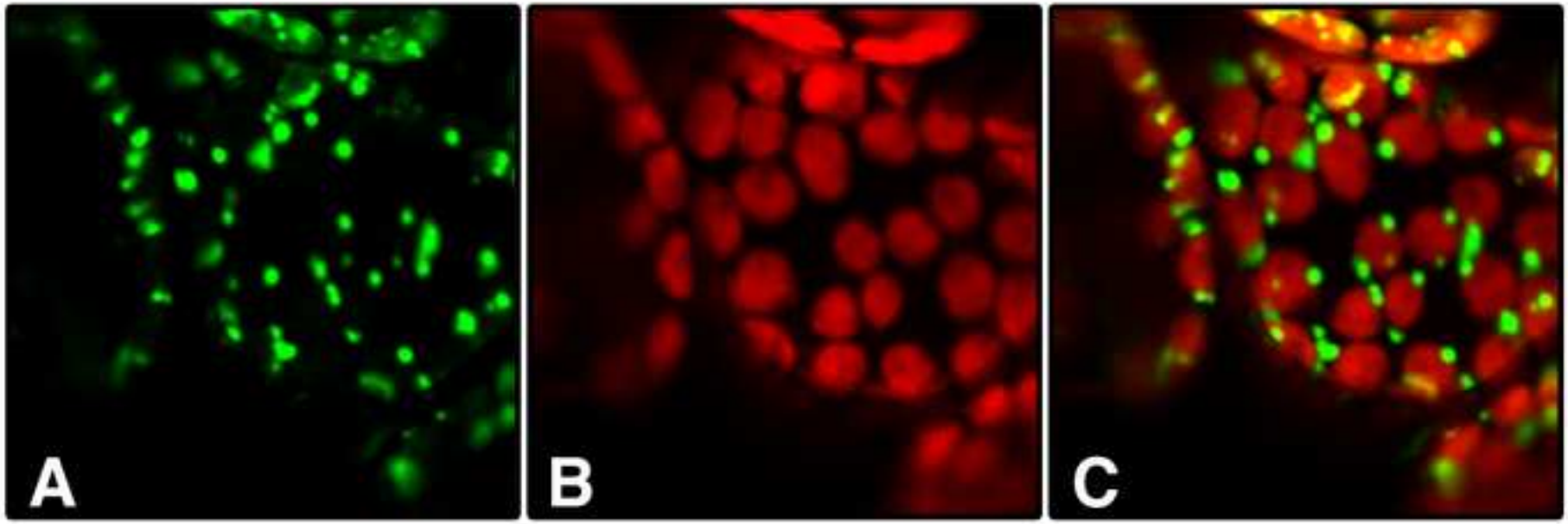

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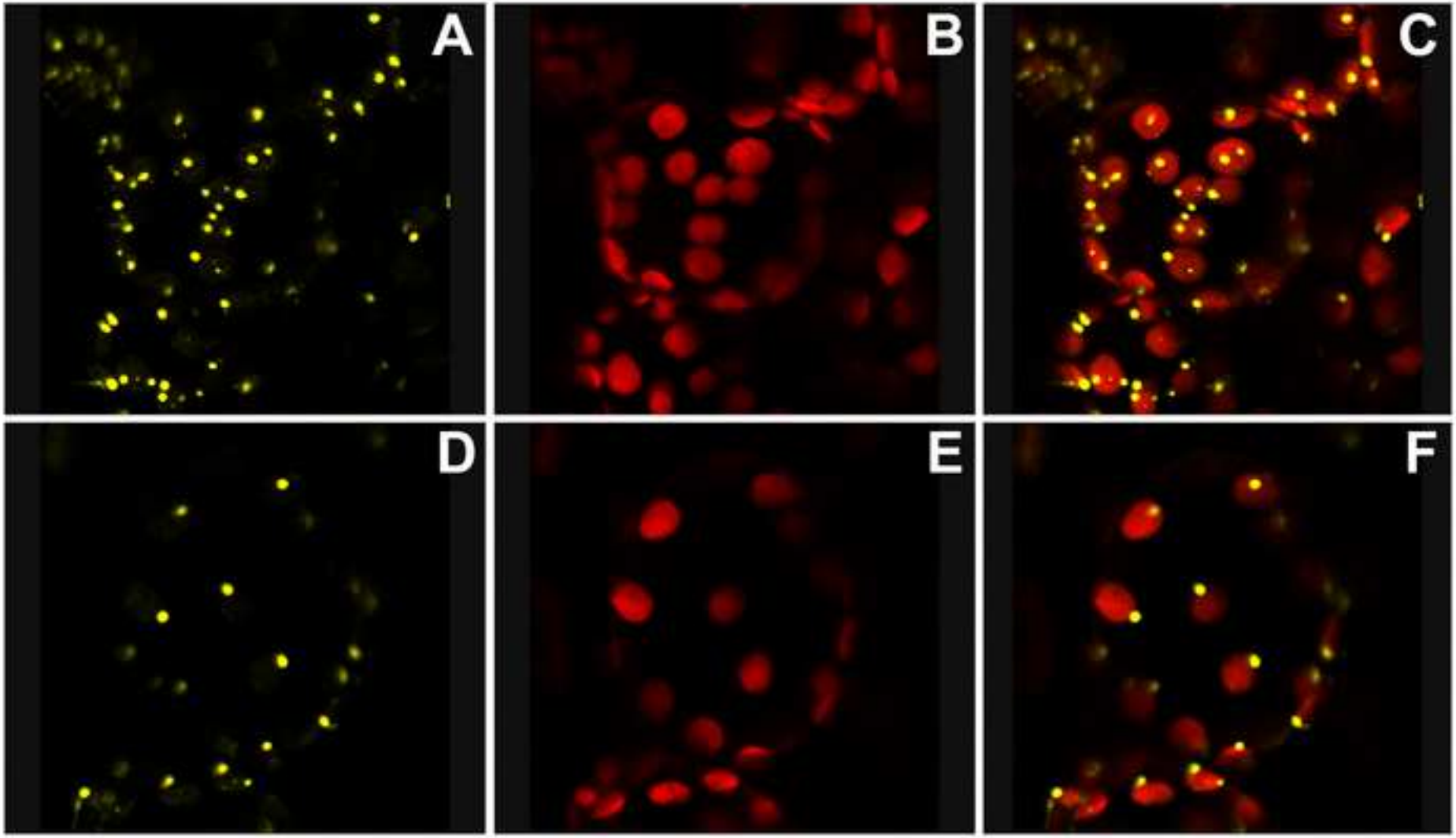

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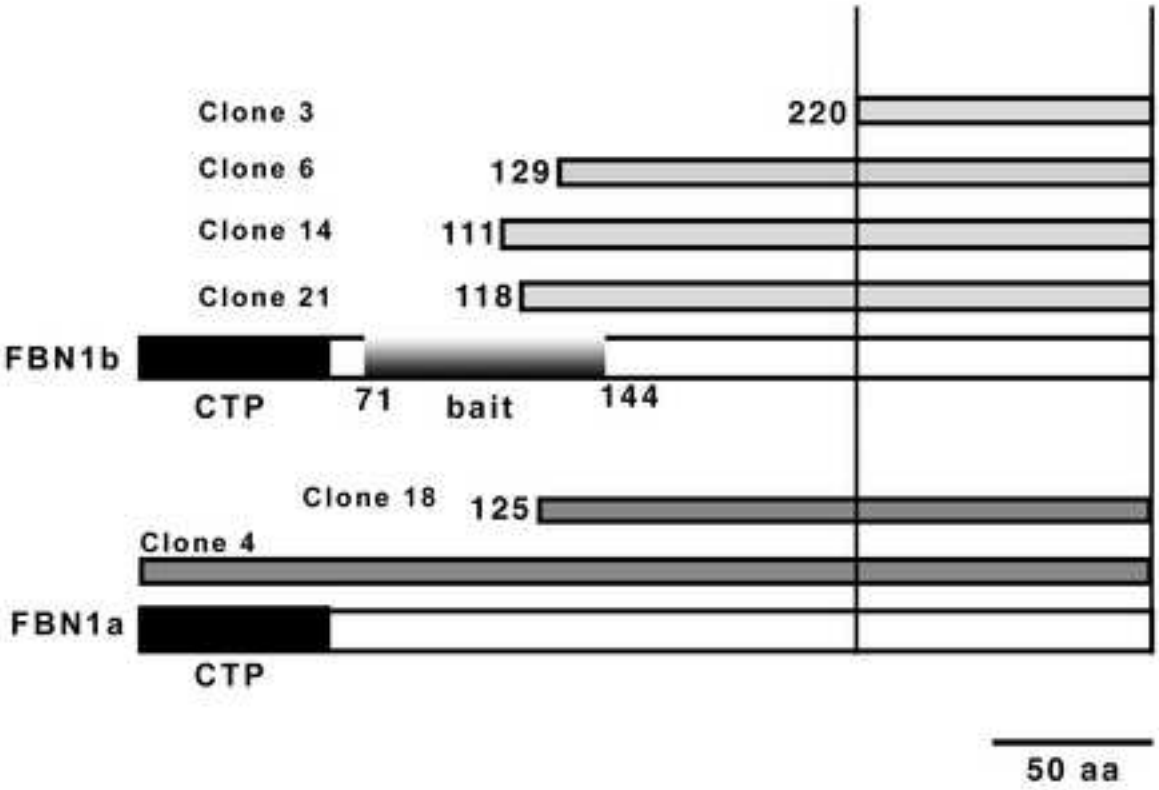

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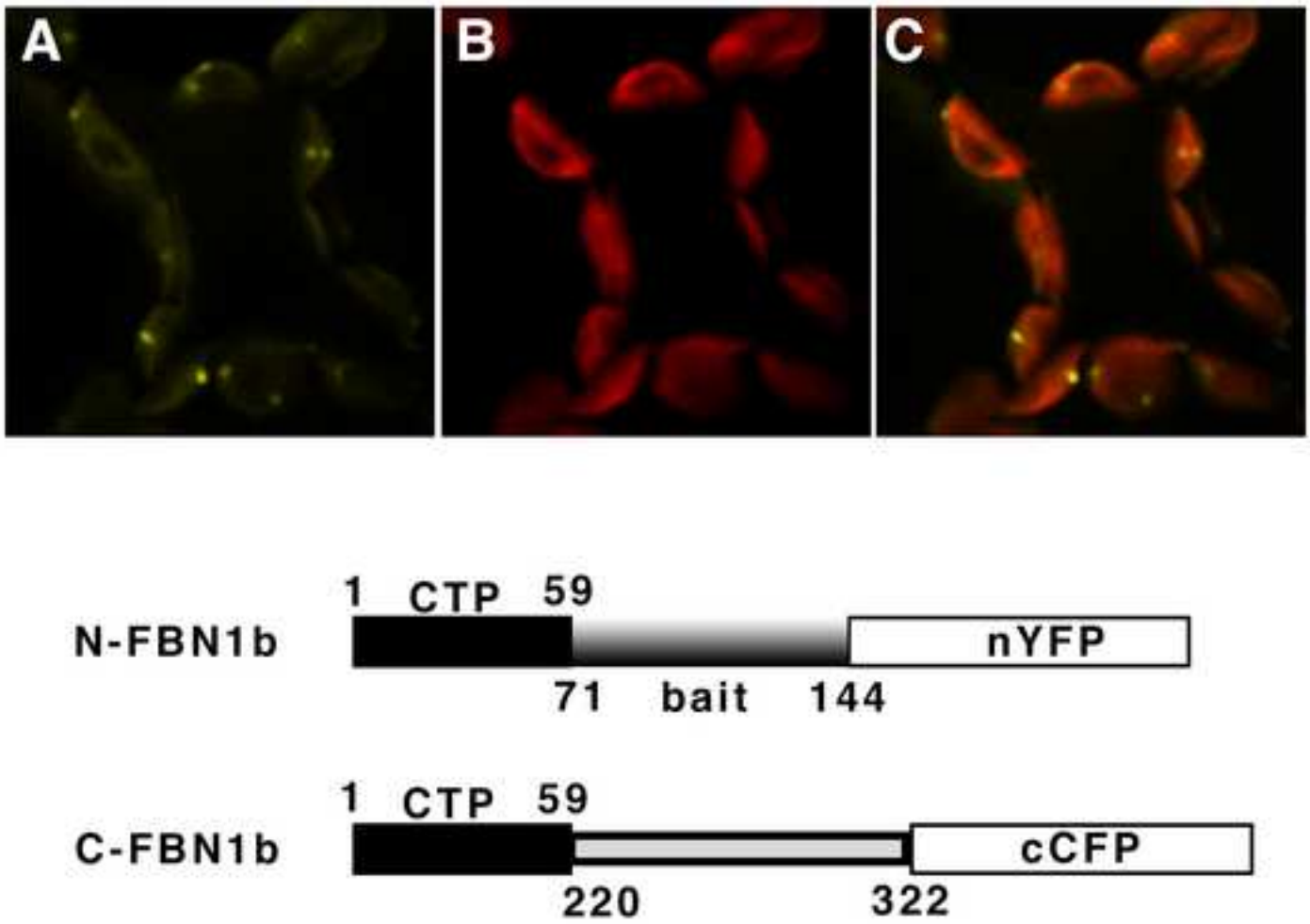

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